Journal of Hazardous Materials 286 (2015) 269–275

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Enhanced tolerance and remediation to mixed contaminates of PCBs and 2,4-DCP by transgenic alfalfa plants expressing the 2,3-dihydroxybiphenyl-1,2-dioxygenase

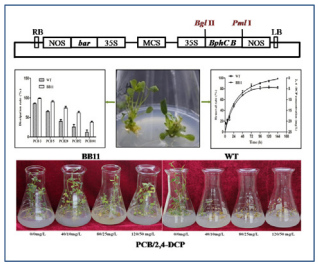
Yan Wang[a,b], Hejun Ren[a,*], Hongyu Pan[b], Jinliang Liu[b], Lanying Zhang[a]

[a] Key Laboratory of Groud Water Resources and Environment of the Ministry of Education, College of Environment and Resources, Jilin University, 2519 Jiefang Road, Changchun, Jilin 130021, People's Republic of China
[b] College of Plant Sciences, Jilin University, 5333 Xi'an Road, Changchun, Jilin 130062, People's Republic of China

HIGHLIGHTS

- Transgenic alfalfa plants expressing the 2,3-dihydroxybiphenyl-1,2-dioxygenase were generated.
- The tolerance capabilities of transgenic alfalfa plants towards mixed contaminants of PCBs and 2,4-DCP became markedly increased.
- Transgenic alfalfa plants exhibited strong dissipation of PCBs.
- High removal efficiency of 2,4-DCP for transgenic alfalfa plants was reached in a short time.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 23 July 2014
Received in revised form
14 November 2014
Accepted 24 December 2014
Available online 27 December 2014

Keywords:
Polychlorinated biphenyls
2,4-Dichlorophenol
2,3-Dihydroxybiphenyl-1,2-dioxygenase
Transgenic alfalfas
Phytoremediation

ABSTRACT

Polychlorinated biphenyls (PCBs) and 2,4-dichlorophenol (2,4-DCP) generally led to mixed contamination of soils as a result of commercial and agricultural activities. Their accumulation in the environment poses great risks to human and animal health. Therefore, the effective strategies for disposal of these pollutants are urgently needed. In this study, genetic engineering to enhance PCBs/2,4-DCP phytoremediation is a focus. We cloned the 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC.B) from a soil metagenomic library, which is the key enzyme of aerobic catabolism of a variety of aromatic compounds, and then it was expressed in alfalfa driven by CaMV 35S promoter using *Agrobacterium*-mediated transformation. Transgenic line BB11 was selected out through PCR, Western blot analysis and enzyme activity assays. Its disposal and tolerance to both PCBs and 2,4-DCP were examined. The tolerance capability of transgenic line BB11 towards complex contaminants of PCBs/2,4-DCP significantly increased compared with non-transgenic plants. Strong dissipation of PCBs and high removal efficiency of 2,4-DCP were exhibited in a short time. It was confirmed expressing BphC.B would be a feasible strategy to help achieving phytoremediation in mixed contaminated soils with PCBs and 2,4-DCP.

1. Introduction

Human activities and industrialization development generate large amounts of chemicals that are often introduced into soil and water environment. Prevalent contaminants include petroleum hydrocarbons (PHC), polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, pesticides, solvents, metals, and salts. Among them, halogenated hydrocarbons, such as polychlorinated biphenyls (PCBs) and chlorophenols are persistent environmental pollutants [1,2].

PCBs are a class of synthetic organic compounds characterized with high hydrophobicity, toxicity, and bioaccumulation [3]. From

* Corresponding author. Tel.: +86 431 8850 2606; fax: +86 431 8850 2606.
E-mail address: renhejun@126.com (H. Ren).

http://dx.doi.org/10.1016/j.jhazmat.2014.12.049
0304-3894/

1940s to 1980s, commercial PCBs had been widely used. Though production bans, PCBs remain a ubiquitous contaminant worldwide due to extremely slow biodegradation for semi-volatile, low solubility in water, and high tendency of soils or sediments adsorption [4]. 2,4-DCP is recognized as a priority pollutant in aquatic environment in the United States as well as in China due to its high toxicity to aquatic life and resistance to degradation. The presence of 2,4-DCP in soil is generally due to their use as antifungal agents by the industry or the degradation of certain herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D), which widely used for controlling broadleaf weeds in crops and grasses [5]. As a consequence, PCBs and 2,4-DCP had lead to the mixed contamination of soils in general. Therefore, effective strategies are needed for remediation of PCBs and 2,4-DCP in environment.

Comparing with physical and chemical methods, phytoremediation is now emerging as a promising strategy and attracting much attention due to its advantages of being less expensive (the fact that it is carried out *in situ*), environmentally sustainable, and aesthetically pleasing [6]. Nevertheless, many studies have shown the removal rate of PCBs or 2,4-DCP using conventional plants is inadequate and slow [7,8]. The primary reason is that plants lack the necessary enzymatic machinery involved in bacteria or mammals for efficient cleavage of aromatic structure. [9]. Alternatively, there is increasing in the efficiency of phytoremediation, which will be greatly enhanced by transgenic plants bearing bacterial genes involved in xenobiotic metabolism, leading to a wider application in the field [10].

Plants express some exogenous detoxifying enzymes, mainly monooxygenases and peroxidases helping in remediation. Central to PCBs or chlorophenols degradation processes is the opening of thermodynamically stable benzene rings by extradiol dioxygenases, such as 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC) [11]. BphC found in a range of gram-negative and gram-positive bacteria that aerobically assimilate biphenyl, could utilize nonheme ferrous iron to cleave the aromatic nucleus of catechols meta (adjacent) to the yellow substance. BphC is involved in aerobic catabolism of a variety of aromatic compounds including phenol, naphthalene, and polychlorinated biphenyls. Thus, potential utility of BphC is of considerable interest due to its general metabolic significance in the degradation of environmental pollutants [12]. However, the success of phytoremediation depends upon catabolic genes morphological and intrinsic properties of the plant species themselves. Therefore, choice of plant species is likely to impact on the success of the rhizoremediation technology [13]. *Arabidopsis* and *Nicotiana* are model research organisms and suitable transgenic receptor, but not well adapted for phytoremediation applications, given their small stature and shallow root system [14].

Alfalfa (*Medicago sativa* L.) is one of the most important perennial forage crops all over the world with a variety of elite characteristics, such as highly productive biomass, drought tolerance, fast growing, and available in large amount during several months of the year [15]. Recent study showed transgenic alfalfa had strong ability to decontaminate heavy metal and organic pollutants-contaminated soil. Transgenic alfalfa expressing a modified bacterial Atrazine chlorohydrolase (AtzA) could endure over a wide range of atrazine concentrations [16]. It was found transgenic alfalfa co-expressing glutathione S-transferase and human P450 2E1 enhanced phytoremediation of mixed heavy metal (mercury or cadmium)-organic pollutants (trichloroethylene) compared to negative control [17,18]. In this sense, alfalfa is an ideal natural resource for remediation of contaminated soils. Whereas, there are scarcely reports about transgenic alfalfa plants designed for phytoremediation of mixed contaminated soil with PCBs and 2,4-DCP. In the present study, we attempted to generate transgenic alfalfa plants expressing bacteria 2,3-dihydroxybiphenyl-1,2-dioxygenase and investigate performance of transgenic plants towards mixed contaminates of PCBs and 2,4-DCP.

2. Materials and methods

2.1. Chemicals and plants

Five polychlorinated biphenyls congeners (IUPAC numbers: PCB 3, 5, 28, 52, and 101) were chosen as exposure for representing the contamination condition and congener difference, because PCB 28, 52, and 101 had been chosen as indicators in Europe and were present in most PCB-mixtures and in the environmental samples [2,19]. As well as, PCB 5 was one of the main components of PCBs once used in China [20]. All above PCB standards were purchased from AccuStandard Inc., USA. Stock standard solutions of PCBs were diluted in hexane and stored at 4 °C until use. 2,4-DCP was purchased from Sigma–Aldrich with purity of 97%. Alfalfa seeds (*Medicago sativa* L. cv. Gongnong No. 1) were kindly provided by Yanzhi Liu (Jilin Academy of Agricultural Sciences, China).

2.2. Cloning of the BphC gene from a metagenomic library

To obtain 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC) gene, a metagenomic library was constructed from a PCBs-contaminated soil sample, as described by our previous study [21]. Functional screening of BphC was performed by spraying the agar plates containing 20 mg/mL 2,3-dihydroxybiphenyl solution (dissolved in acetone). Transformed colonies formed a yellow halo within 30 s, indicating the cleavage of 2,3-dihydroxybiphenyl, were selected as positive clones. The nucleotide sequences of the inserts from selected colonies were further obtained by sequencing. A putative 2,3-dihydroxybiphenyl-1,2-dioxygenase gene (designated as BphC.B) was directly amplified by PCR and then ligated into *Nde* I and *Bam* HI-digested plasmid pET28a (Novagen, USA). The positive vector was transformed into *E. coli* BL21 (DE3) pLysS for overexpression. The expressed protein was then affinity purified using a Ni-NTA column (Qiagen, Germany) according to Lu et al. [22]. The molecular mass of denatured recombinant protein and the purity of recombinant protein were determined by using 12% SDS-PAGE. Western blot analysis of purified recombinant protein was performed with mouse anti-6× His monoclonal and HRP-conjugate goat anti-mouse IgG antibodies (Beijing Biosynthesis Biotechnology Co., Ltd., China). The substrate specificity was subsequently determined by the formation of ring meta-cleavage products using catecholic compounds: catechol, 375 nm (ϵ = 33 000 M^{-1} cm^{-1}); 3-methylcatechol, 388 nm (ϵ = 13 400 M^{-1} cm^{-1}); 4-methylcatechol, 379 nm (ϵ = 28 100 M^{-1} cm^{-1}); 4-chlorocatechol, 379 nm (ϵ = 36 100 M^{-1} cm^{-1}); and 2,3-dihydroxybiphenyl, 434 nm (ϵ = 13 200 M^{-1} cm^{-1}) [23]. The standard enzyme activity assays were performed by incubating the purified enzyme (50 μg) with 50 μM compounds in 50 mM sodium phosphate buffer (pH 7.5) at 25 °C in 1 mL. The production was monitored in a UV–vis spectrophotometer (Shimadzu Co., Japan). One unit of activity was defined as the amount of enzyme catalyzes the formation of 1 μmol of the product per minute at 25 °C. All experiments were performed in quadruplicate.

2.3. Transformation and selection of alfalfa transformants lines

The gene fragment coding BphC.B was inserted into a plant binary expression vector pCAMBIA 3301 by replacing the GUS gene between CaMV 35S promoter and terminator (Fig. S1). The p3301-BphC.B and null p3301 plasmids were introduced

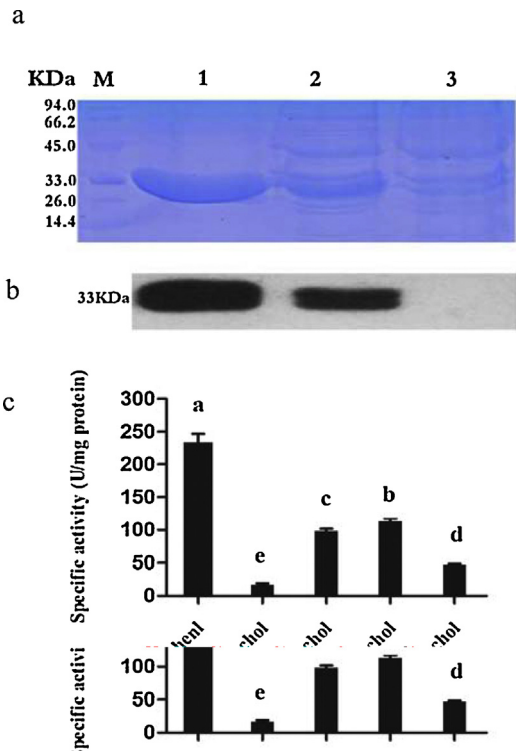


Fig. 1. Characteristic features of BphC.B expressed in *E. coli*.
(a) BphC.B was separated by 12% (w/v) SDS-PAGE and then stained with Coomassie brilliant blue G-250. M, protein molecular weight markers (Tiangen); Lane 1, affinity purified target protein of BphC.B; Lane 2, unpurified target protein of BphC.B from supernatant of cell lysates; and Lane 3, supernatant of the cell lysates with pET-28a (+).
(b) Western blot analysis of BphC.B protein. Proteins were loaded on 12% SDS-PAGE and analyzed by Western blot using the anti-His antibody.
(c) Substrate specificity of BphC.B on catecholic compounds. The standard enzyme activity assays were performed by incubating the purified enzyme (50 μg) with 50 μM compounds in 50 mM sodium phosphate buffer (pH 7.5) at 25 °C in 1 mL. The production was monitored in a UV–vis spectrophotometer. Values presented are means ± SD of three replicates.

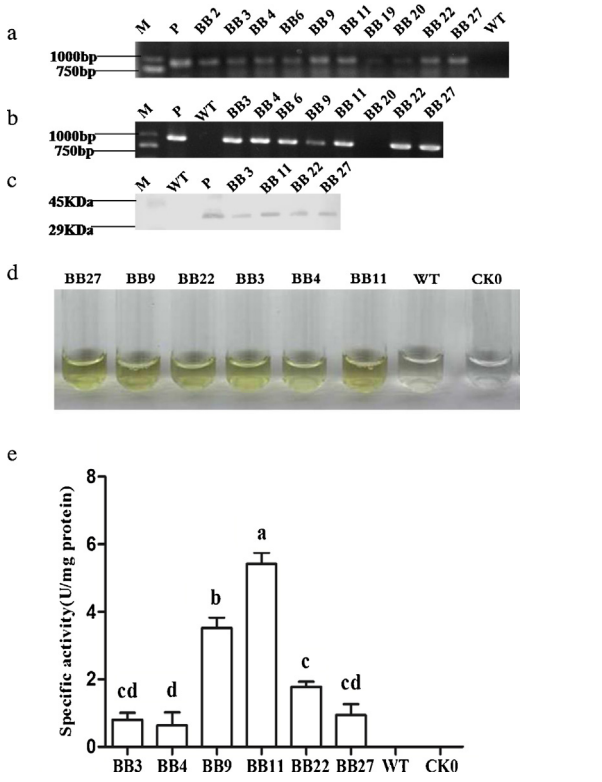


Fig. 2. Molecular charaterization and enzyme activity analysis of transgenic alfalfa plants.
(a) Screening of transgenic alfalfa lines for the presence of BphC.B gene (900 bp) through PCR analysis in the leaves from ten independent putative transgenic plants. BB3–BB27 represents transgenic alfalfa lines transformed with p3301–BphCB; P, plasmid p3301–BphCB as a positive control; WT, wild-type alfalfa as a negative control; and M, DNA size markers.
(b) RT–PCR analysis of BphC.B gene in leaves from transgenic lines. No BphC.B transcript was detected in wild-type (WT) plants.
(c) Western blot analysis was performed on protein samples of wild-type and transgenic lines. Proteins from selected transgenic and control lines were loaded on SDS-PAGE and analyzed by Western blot using the BphC.B polyclonal antibody; M, prestained protein size markers.
(d) Yellow cleavage product (HOPDA) of 2,3-dihydroxybiphenyl in crude extracts of transgenic alfalfa plants.
(e) Cleavage activities for 2,3-dihydroxybiphenyl in crude extracts of transgenic alfalfa. The activities are shown as the means and standard deviations of measurements from three independent experiments. CK0 represents transgenic alfalfa plants transformed with the null vector pCAMBIA3301; WT represents wild-type alfalfa; Lines (BB3–BB27) represent transgenic alfalfa plants expressing BphC.B.

into *Agrobacterium tumefaciens* EHA105 by freeze-thaw method and subsequently transformed into alfalfa using the leaf-dipping method [24]. Regenerated shoots from embryos with 3–5 roots were selected on solid MS medium containing Basta (Phosphinothricin) at 2 mg/L for 10 days and then transplanted on solid MS media containing timentin 200 mg/L (Fig. S2). Asexual reproductions of transgenic alfalfa lines (T_0) were carried out by the cutting method for continuous passage culture.

2.4. Molecular analysis of transgenic plants

DNA was extracted from alfalfa leaves of Basta resistant T_0 transformants and then PCR was performed to confirm positive transgenic plants by using a pair of BphC.B gene specific primers: YF: 5′-GATGGTAGCAGTCACGGAAC-3′ and YR: 5′-TCAGCCGAGGGGAATATCCAT-3′. RT–PCR was also performed to validate the expression of BphC.B in transcript level. In order to detect the expression of BphC.B, rabbits were immunize with purified recombinant His–BphC.B to prepare polyclonal antiserum [25]. The titer and specificity of polyclonal antiserum developed against recombinant BphC.B were evaluated by immune double gel diffusion and enzyme-linked immune sorbent assay (ELISA). Blot analysis was performed to detect the expression of BphC.B in transgenic alfalfa plants [26].

The enzyme activity of BphC.B in crude plant extracts toward 2,3-dihydroxybiphenyl was also assayed by measuring the absorption value at 434 nm for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) according to above described method. One unit of activity was defined as the amount of enzyme required to generate 1 μmol of HOPDA per minute.

2.5. PCBs dissipation assays

The mixed-PCBs were solubilized in *N*,*N*-dimethylformamide (DMF) and then added to a total of 200 mL 1/2 MS medium in each flask to a final concentration of 0.015 mg/L for each congener. Transgenic alfalfa lines and wild-type alfalfas with similar biomass were grown in 1/2 MS medium containing mixed-PCBs in triplicates. After 7 days, medium and plants were sampled. The effect on growth induced after 7 days of exposure were estimated by determining fresh weight of plant and tolerance index [27].

2.6. 2,4-DCP removal assays

The 2,4-DCP removal rates of wild-type and transgenic alfalfa plants in hydroponic conditions were measured. Four seedlings with similar size were transferred to 100 mL glass flasks with 1/2

MS medium containing 25 mg/L of 2,4-DCP. A negative control was performed using 1/2 MS medium without 2,4-DCP. The seedlings were incubated at 25 °C. The solution samples were taken periodically (2, 6, 12, 24, 48, 72, 96, 120, and 144 h) for residual 2,4-DCP determination. The removal efficiency was defined as percentage of the pollutant removed from the solution. After 6 days, their wet mass was recorded to evaluate the tolerance to 2,4-DCP. Two independent experiments were carried out.

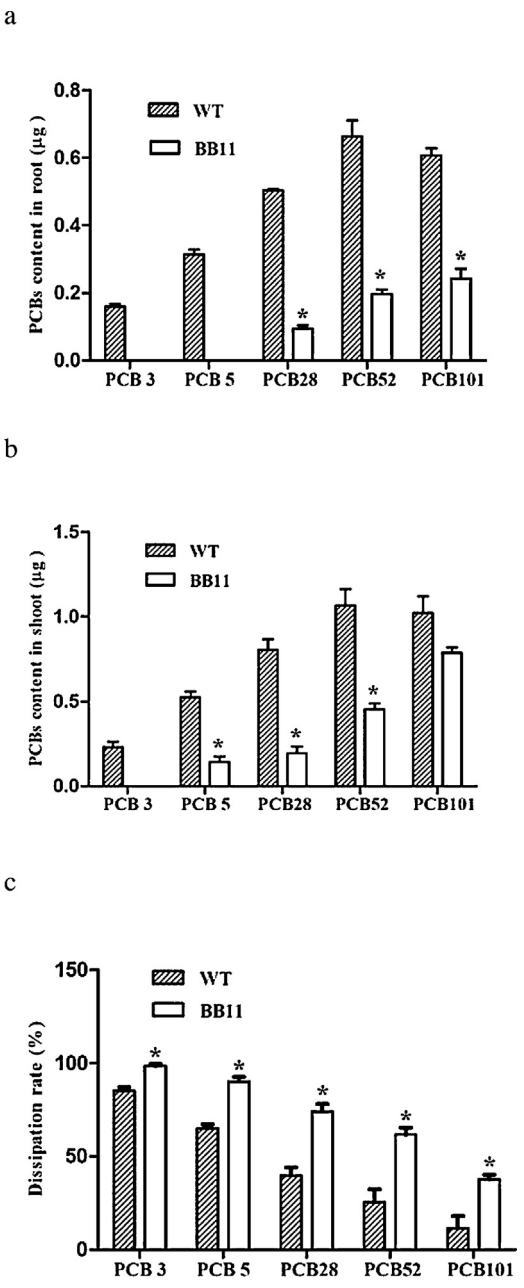


Fig. 3. Dissipation of transgenic alfalfa to PCBs.
Transgenic alfalfa and wild-type alfalfa plants were grown for 7 days on 1/2MS hydroponic medium containing 0.075 mg/L PCBs and then measure content of PCBs in root (a), shoot (b) of alfalfa, and dissipation rate of PCBs (c). WT represents wild-type alfalfa; BB11 represents transgenic line BB11.

$$\text{Dissipation rate (\%)} = \frac{\text{initial content of PCBs} - \text{content of PCBs that extracted from medium, shoot and root}}{\text{initial content of PCBs}} \times 100$$

All the values are means of three replicates ± S.E. and significant differences between wild-type alfalfas and transgenic alfalfas as determined by *t*-test at $p \leq 0.05$ are indicated by one asterisk (SAS 8.0).

2.7. PCBs/2,4-DCP tolerance assays

To further evaluate PCBs/2,4-DCP tolerance of transgenic alfalfa lines, PCB 5 was selected for testing as it was one of the main components of PCBs once used in China [20], and three seedlings of transgenic lines and wild-type alfalfa plants that had similar biomass were cultured on MS solid medium amended with PCB 5/2,4-DCP to various final concentrations of 40/10, 80/25, and 120/50 mg/L, respectively. After 4 weeks, pictures of transgenic lines were taken. The concentrations of these toxic pollutants were confirmed to damage the control alfalfa plants in preliminary experiments.

2.8. Measurements of PCBs and 2,4-DCP by GC–MS and Spectrophotometer

For determining PCB congener contents in seedlings of exposure experiment, plant samples (divided into shoots and roots) were ground with a mortar to fine powder after dried at 60 °C for 12 h, and the powder were all transferred into conical flask with 20 mL 1:1 (v/v) solvent of acetone and hexane, then extracted by ultrasonication for 20 min [28]. The extract was passed through an anhydrous Na_2SO_4 column with elution of hexane into round bottom flask. Solvents were evaporated by a rotary evaporator and resuspended in 500 μL hexane. Analysis was run in a GC–MS (6890/5973, Agilent, USA) equipped with a DB–5MS capillary column (30 m × 0.25 mm × 0.25 um). The MS was operated in selected ion monitoring mode (SIM). PCB congeners were monitored at the two most intense ions of the molecular cluster. Quantification of PCBs was based on external calibration standards containing known concentration of PCB congeners (PCB 3, 5, 28, 52, and 101). Mixed standards with known amount of the PCB congeners were added to 1/2 MS medium and plant tissues before extraction for assessment of recovery rates. Average percentage recovery for the individual selected PCB congeners from 1/2 MS medium and plant tissues varied from 85 to 110% and from 90 to 116%, respectively ($n = 7$, RSD < 10%). Blanks covering the entire analytical procedure (from the extraction to GC analysis) were analyzed and did not reveal any PCB contamination. The method detection limits (MDLs) for PCB congeners from 1/2 MS medium and plant tissues were in ranges of 0.63–0.91 μg/kg and 0.32–0.61 μg/kg.

To measure the concentration of 2,4-DCP, aliquots of 600 μL of each sample were mixed with 100 μL of 4-aminoantipyrine (20.8 mM), 100 μL potassium ferricyanide (83.4 mM), and 200 μL of sodium bicarbonate (0.25 M/pH 8.4). Incubation 5 min at room temperature, absorbance of the colored extract was determined at 510 nm by a spectrophotometer [1].

In all experiments three replicates were performed for each sample. All the data were subjected to one way ANOVA using the software EXCEL. Results were presented with the mean and the standard error. Activity of BphC.B enzymes were analyzed by Duncan's multiple comparison tests and other data were analyzed by the *t*-test. Statistical significance was set to $p < 0.05$ (SAS 8.0).

3. Results

3.1. Identification and characterization of BphC.B

A metagenomic plasmid library was constructed using PCBs-contaminated soil samples. One of the transformants turned yellow was selected out. Sequence analysis of the insert DNA (GenBank accession no. GQ403988.1) showed the presence of

Table 1
Fresh weight of wild-type and transgenic alfalfa seedlings after exposure to PCBs or 2,4-DCP.

Pollutants	Concentration (mg/L)	Wild-type alfalfa				Transgenic alfalfa BB11			
		Weight before exposure (g)	Weight after exposure (g)	Weight gain/loss (g)	TI (%)	Weight before exposure (g)	Weight after exposure (g)	Weight gain/loss (g)	TI (%)
PCBs	0	1.60 ± 0.10	1.68 ± 0.28	0.08 ± 0.02	100	1.59 ± 0.15	1.66 ± 0.15	0.07 ± 0.02	100
	0.075	1.52 ± 0.17	0.89 ± 0.14	−0.63 ± 0.30	54	1.51 ± 0.18	1.47 ± 0.17	−0.04 ± 0.01	88*
2,4-DCP	0	1.78 ± 0.13	1.83 ± 0.07	0.03 ± 0.10	100	1.78 ± 0.08	1.81 ± 0.06	0.03 ± 0.02	100
	25	1.81 ± 0.17	1.4 ± 0.10	−0.39 ± 0.04	77	1.80 ± 0.20	1.77 ± 0.13	−0.03 ± 0.07	98*

Tolerance index (TI) = fresh weight of pollutants treated seedlings/fresh weight of pollutants untreated seedlings.
All the values are means of three replicates ± S.E. and significant differences from wild-type alfalfas as determined by *t*-test are indicated by one asterisk.

one complete putative ORF, which contained a 900 bp nucleotide sequence and encoded a 299 amino acids protein with a predicted molecular weight of 33 kDa. PSI-BLASTP analysis reveals that its deduced amino acid sequence was highly similar to other catechol 2,3-dioxygenase or 2,3-dihydroxybiphenyl-1,2-dioxygenase, commonly called as extradiol dioxygenases (EDOs). The predicted protein sequence of the ORF exhibited the highest identity (100%) to a putative 2,3-dihydroxybiphenyl-1,2-dioxygenase (GenBank accession no. YP_004128522) of *Sphingobium yanoikuyae* strain B1. Therefore, it was designated as BphC.B. The purified enzyme migrated as a single band with a molecular weight of about 33 kDa on SDS-PAGE, close to its theoretical molecular weight (Fig. 1a). Western blot analysis with an anti-His antiserum also showed a single band in PVDF membrane (Fig. 1b). As shown Fig. 1c, BphC.B showed higher catalytic activity for 2,3-dihydroxybiphenyl and lowest activity for catechol. It cleaved dihydroxylated substrates in the following order in terms of specificity: 2,3-dihydroxybiphenyl > 4-methylcatechol > 3-methylcatechol > 4-chlorocatechol > catechol. This implied 2,3-dihydroxybiphenyl was the best one catalyzed by the enzyme in the given substrates and had a wide range of substrates, which might be helpful for catalyzing the opening of a variety of the thermodynamically stable benzene rings.

3.2. Characterization of transgenic alfalfa plants

To confirm the present of BphC.B gene in transgenic alfalfa plants, total DNA and RNA from the seedlings were extracted, respectively. DNA–PCR and RT–PCR analysis were subsequently performed. As expected, a 0.9-kb DNA product was detected in the Basta-resistant independent transgenic alfalfas (BB3, BB4, BB9, BB11, BB22, and BB27), but not in wild-type (Fig. 2a and b). To confirm the expression of BphC.B from corresponding integrated genes, Western blot of transgenic alfalfa plants was carried out with the prepared rabbit antibodies. An obvious protein band about 33 kDa, in accordance with the expected molecular weight of BphC.B protein, was detected in the seedling extracts prepared with transgenic alfalfa BB3, BB11, BB22, BB27, and positive control (His-tagged BphC.B fragment expressed in *E.coli*), but not in wild-type alfalfa plants (Fig. 2c).

The BphC.B activity toward 2,3-dihydroxybiphenyl in transgenic alfalfa plants was checked by measuring the amount of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA). The yellow ring-fission products (HOPDA) were produced by transgenic alfalfa plants when incubated with 2,3-dihydroxybiphenyl (Fig. 2d). All transgenic alfalfa plants showed higher BphC.B activity than wild-type plants. The enzyme activities in transgenic lines ranged from 0.64 to 5.42 U/mg. It is noteworthy BphC.B activity in transgenic line BB11 was the highest among transgenic alfalfa plants (Fig. 2e). Thus, transgenic line BB11 was employed to carry out tolerance assay.

3.3. Tolerance and dissipation in transgenic alfalfa to PCBs

Transgenic line BB11 with healthy growth and high activity of BphC enzyme was chosen for removal efficiency of plants to PCBs. The effect of BphC.B expression on PCBs tolerance of transgenic

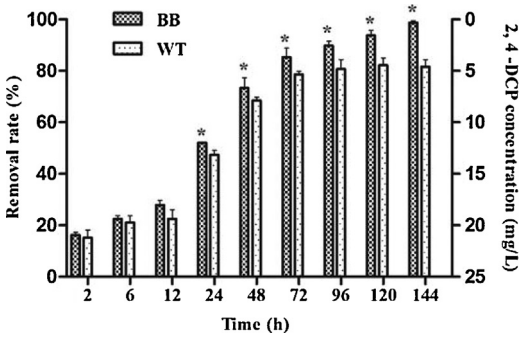


Fig. 4. Removal rates and concentrations in post removal solution of 2,4-DCP at 25 mg/L.
Plants were grown in a 1/2MS hydroponic medium and then transferred to 25 mg/L 2,4-DCP during 7 days. 2,4-DCP concentrations were expressed as milligram per liter and their removal rates were calculated from the remaining 2,4-DCP in the medium. Data are means ± SD of two independent determinations. WT represents wild-type alfalfa; BB11 represents transgenic line BB11.

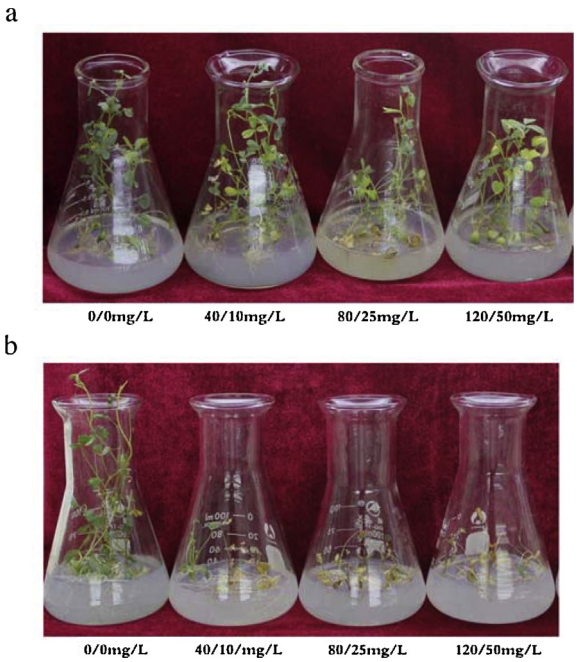


Fig. 5. Transgenic alfalfa plant tolerance to PCB 5 and 2,4-DCP.
Transgenic alfalfa plant BB11 (a) and wild-type alfalfa plants (b) with PCB 5/2,4-DCP at 0/0, 40/10, 80/25, and 120/50 mg/L, respectively. PCB/2,4-DCP tolerance in transgenic alfalfa lines was observed as plant growth.

alfalfa plants was evaluated by monitoring fresh weight and tolerance index of the seedlings after 1 week of exposure to PCBs at 25 °C. Based on the results of biomass production (Table 1), alfalfa plants had no growing effect in 1/2 MS medium without PCBs after introduction of BphC.B. However, after exposed to PCBs, transgenic seedlings had a better performance in terms of growing and its tolerance index was 34% higher than the non-transgenic ones.

Remediation efficiency of transgenic line BB11 to PCBs was evaluated in hydroponic assay. PCBs contents of wild-type and transgenic alfalfa lines were determined and normalized to fresh weight. The results indicated PCBs content in shoots and roots of all transgenic lines were lower than that in wild-type alfalfa plants. The content of PCBs in roots and shoots in wild-type alfalfas were 1.29–5.28 times higher than transgenic lines (Fig. 3a and b). Furthermore, PCB 3 was not detected in shoots of transgenic lines. Similarly, both PCB 3 and PCB 5 could be absent in roots in transgenic lines. The dissipation rates of PCBs in transgenic lines were higher than wild-type alfalfas (Fig. 3c). It indicated transgenic lines showed significant improvement of PCBs degradation rate compared with the controls.

3.4. Tolerance and removal in transgenic alfalfa to 2,4-DCP

To evaluate 2,4-DCP tolerance conferred by expression of BphC.B, seedlings of transgenic, and wild-type alfalfa plants were planted directly in 1/2 MS medium with 2,4-DCP at 25 mg/L. Total biomass production and tolerance index were determined on surviving transgenic and wild-type alfalfa seedlings. As shown in Table 1, no significant difference was found between total biomass production of the wild-type and transgenic alfalfa plants in liquid medium without 2,4-DCP. In contrast, when subjected to 25 mg/L 2,4-DCP treatment, biomass, and tolerance index of transgenic seedlings were significantly higher than that of wild-type alfalfas. Meanwhile, the removal efficiency of transgenic alfalfa line BB11 was evaluated in a short time (Fig. 4). During 2–12 h of treatment, removal efficiency was lower than 30% and showed no significant differences between wild-type and transgenic plants. After 48 h, removal efficiency of 2,4-DCP for transgenic plants had been higher than wild-type plants. Removal efficiency of transgenic lines showed an increasing trend after 72 h of treatment however no obvious changes for wild-type plants. Removal efficiency of the transgenic plants reached its plateau at 98.8% at the time point of 144 h after treatment. The *t*-test showed removal efficiency of transgenic plants was significantly higher than wild-type alfalfas.

3.5. Combination PCB/2,4-DCP tolerance in transgenic alfalfa plants

Tolerance to the combination of PCB 5 and 2,4-DCP was assayed using transgenic plant BB11 planted in glass flasks for 4 weeks. As seen in Fig. 5, no visual evidence of damage was observed for transgenic plant BB11 and it exhibited strong resistance up to 120/50 mg/L of PCB 5/2,4-DCP complex contaminants. Transgenic plants thrived well in the coexistence of PCB 5 (80 mg/L) and 2,4-DCP (25 mg/L), whereas, non-transgenic control plants showed marked symptoms of severe damage possibly induced by the complex pollutants and were almost dead. As expected, the transgenic alfalfa BB11 expressing BphC.B was found to show cross-tolerance to PCBs and 2,4-DCP in vegetative growth stages.

4. Discussion

In the present study, we sought to develop transgenic plants used for phytoremediation of soil polluted with PCBs and 2,4-DCP. We thus, genetically engineered alfalfa plants to express a BphC.B from soil metagenome that showed a wide substrate utilization spectrum (Fig. 1). Abundant transgenic alfalfa plants were obtained by *Agrobacterium*-mediated transformation approach, and identified by molecular technologies. What is more, no considerable morphological abnormalities were observed in all the transgenic lines, which grew vigorously at similar rates to the wide-type in MS medium (data not shown). Among transgenic lines, the highest enzyme activity was detected in transgenic line BB11 (Fig. 2e). It might be possibly caused by different insertion sites that affect expression levels. This variation is common in transgenic plants.

In general, cleaning up pollution with wild-type plants lead to the accumulation of PCBs in shoots and roots of plants that may be released to the soil or volatilize into atmosphere [28,29]. However, in transgenic plant BB11, the content of PCB congeners in both shoots and roots were significantly lower than that of the corresponding wild-type controls. In particular, PCB 3 could not be detected in shoot and root in transgenic lines and also PCB 5 could not be detected in root (Fig. 3a and b). Transgenic alfalfa BB11 showed an enhanced ability to remove PCBs from medium as compared to untransformed controls. Of the total dissipation rates, the percentage of PCBs in the transgenic plants was 12–36% higher than that in wild-type lines (Fig. 3c). It can be attributed to that metabolism of hydroxylated chloro-biphenyl by BphC.B could alleviate toxic effect to transgenic lines, while wild-type lines without BphC.B would be severely affected by its toxicity. This was consistent with results of alfalfas tolerance index (Table 1). Due to detoxification effect, transgenic alfalfa plants could grow healthily so that they continuously uptake and dissipate a large amount of PCBs with the biomass increase. This is in agreement with the requirements for successful *in situ* phytoremediation, plants need (a) to have tolerance towards the chemicals or pollutants to be cleaned, (b) to have the ability to metabolize and immobilize them, and (c) to have a large biomass so that they can remediate widespread chemicals in the field [30].

As it could be seen in previous reports, 2,4-DCP has great toxicity impact on living organisms. The concentrations of 2,4-DCP from 5 mg/L induce mortality in *Rhinella arenarum* embryos. Moreover, a low concentration of 2,4-DCP (0.97 mg/L) produced a 50% of growth inhibition in the green algae *Pseudokirchneriella subcapitata* when they were used for the toxicity assessment for this pollutant [31]. Transgenic alfalfa BB11 that can remove 2,4-DCP showed better efficiency than wild alfalfa plants in response to 25 mg/L 2,4-DCP after 144 h (Fig. 4). Although there was a minor increase of 17% in removal efficiency for 25 mg/L 2,4-DCP, the concentration of 2,4-DCP in post removal solutions for transgenic alfalfas was only 0.47 mg/L, much less than the remaining 4.61 mg/L for wild-type alfalfa plants. The high and rapid removal potential of transgenic alfalfa BB11 for 2,4-DCP could be due to the high cleaving activity of foreign BphC.B enzyme for chlorocatechol, which allowing this tissue to remove 2,4-DCP in a short time. Some results from other researches were consistent with these findings. For instance, an increased efficiency for 2,4-DCP removal was also found in using transgenic tobacco overexpressing peroxidase gene [32]. Hence, transgenic alfalfa BB11 plants have the great potential to be used for phytoremediation of 2,4-DCP due to the high efficiency in a short time.

For evaluating tolerance of transgenic alfalfa BB11 to different combination concentrations of 2,4-DCP and PCBs, both transgenic alfalfa BB11 and non-transgenic plants were subjected to increasing concentrations of PCB 5/2,4-DCP as shown in Fig. 5. The tolerance tests revealed transgenic line BB11 was able to sustain growth in PCB 5/2,4-DCP mixed contaminants concentration up to 120/50 mg/L. However, wild-type alfalfa plants can barely withstand 40/10 mg/L. Therefore, transgenic plants showed higher tolerance to complex pollutants especially at high level of PCB 5/2,4-DCP in comparison with wild-type alfalfa plants. It was demonstrated that the tolerance to mixed pollutants of PCB

5/2,4-DCP was considerably improved by expression of BphC.B in transgenic alfalfa plants.

Transgenic phytoremediation trials have resulted in increased dissipation of organic contaminants. To our knowledge, this work represents the first development of transgenic alfalfa plants for the phytoremediation of mixed pollution of PCBs and 2,4-DCP. The studies would give us beneficial enlightenment that these transgenic species would provide a good strategy to tackle the environmental contaminants and mixed organics contaminated problem in particular. It is hoped, in the near future, the utility and efficiency of our newly designed transgenic alfalfa plants could possibly be validated in field experiments. More studies are needed to further understand the processes of PCBs/2,4-DCP uptake, translocation, and rhizosphere effect in transgenic alfalfa plants.

Acknowledgements

This work was supported by Graduate Innovation Fund of Jilin University of China (450060488106), Natural Science Research Foundation of Jilin Province of China (20140520155JH), The National Natural Science Foundation of China (41101226 and 41471252), Specialized Research Fund for the Doctoral Program of Higher Education of China (SRFDP) under grant (20110061120076) and The Fundamental Research Funds for the Central Universitiesunder grant (2013ZY02).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2014.12.049.

References

[1] M.A. Talano, S. Frontera, P. González, M.I. Medina, E. Agostini, Removal of 2,4-diclorophenol from aqueous solutions using tobacco hairy root cultures, J. Hazard. Mater. 176 (2010) 784–791.
[2] S.N. Meijer, W. Ockenden, A. Sweetman, K. Breivik, J.O. Grimalt, K.C. Jones, Global distribution and budget of PCBs and HCB in background surface soils: implications for sources and environmental processes, Environ. Sci. Technol. 37 (2003) 667–672.
[3] I. Danse, R. Jaeger, R. Kava, M. Kroger, W. London, F. Lu, R. Maickel, J. McKetta, G. Newell, S. Shindell, Position paper of the American council on science and health: public health concerns about environmental polychlorinated biphenyls (PCBs), Ecotoxicol. Environ. Saf. 38 (1997) 71–84.
[4] M.L. Whitfield Åslund, B.A. Zeeb, A. Rutter, K.J. Reimer, *In situ* phytoextraction of polychlorinated biphenyl – (PCB) contaminated soil, Sci. Total Environ. 374 (2007) 1–12.
[5] M. Czaplicka, Sources and transformations of chlorophenols in the natural environment, Sci. Total Environ. 322 (2004) 21–39.
[6] U. Krämer, Phytoremediation: novel approaches to cleaning up polluted soils, Curr. Opin. Biotech. 16 (2005) 133–141.
[7] B.A. Zeeb, J.S. Amphlett, A. Rutter, K.J. Reimer, Potential for phytoremediation of polychlorinated biphenyl – (PCB)-contaminated soil, Int. J. Phytoremediat. 8 (2006) 199–221.
[8] T.K. Boucard, R.D. Bardgett, K.C. Jones, K.T. Semple, Influence of plants on the chemical extractability and biodegradability of 2,4-dichlorophenol in soil, Environ. Pollut. 133 (2005) 53–62.
[9] M. Novakova, M. Mackova, Z. Chrastilova, J. Viktorova, M. Szekeres, K. Demnerova, T. Macek, Cloning the bacterial BphC gene into *Nicotiana tabacum* to improve the efficiency of PCB phytoremediation, Biotechnol. Bioeng. 102 (2009) 29–37.
[10] P. Abhilash, S. Jamil, N. Singh, Transgenic plants for enhanced biodegradation and phytoremediation of organic xenobiotics, Biotechnol. Adv. 27 (2009) 474–488.
[11] D.H. Pieper, Aerobic degradation of polychlorinated biphenyls, Appl. Microbiol. Biotechnol. 67 (2005) 170–191.
[12] M. Sylvestre, Prospects for using combined engineered bacterial enzymes and plant systems to rhizoremediate polychlorinated biphenyls, Environ. Microbiol. 15 (2013) 907–915.
[13] M. Sylvestre, T. Macek, M. Mackova, Transgenic plants to improve rhizoremediation of polychlorinated biphenyls (PCBs), Curr. Opin. Biotech. 20 (2009) 242–247.
[14] B. Van Aken, Transgenic plants for enhanced phytoremediation of toxic explosives, Curr. Opin. Biotech. 20 (2009) 231–236.
[15] M.A. Benabderrahim, H. Mansour, F. Ali, Diversity of lucerne (*Medicago sativa* L.) populations in south Tunisia, Pak. J. Bot. 41 (2009) 2851–2861.
[16] L. Wang, D.A. Samac, N. Shapir, L.P. Wackett, C.P. Vance, N.E. Olszewski, M.J. Sadowsky, Biodegradation of atrazine in transgenic plants expressing a modified bacterial Atrazine chlorohydrolase (AtzA) gene, Plant Biotechnol. J. 3 (2005) 475–486.
[17] Y. Zhang, J. Liu, Y. Zhou, T. Gong, J. Wang, Y. Ge, Enhanced phytoremediation of mixed heavy metal (mercury)–organic pollutants (trichloroethylene) with transgenic alfalfa co-expressing glutathione S-transferase and human P450 2E1, J. Hazard. Mater. 260 (2013) 1100–1107.
[18] Y. Zhang, J. Liu, Transgenic alfalfa plants co-expressing glutathione S-transferase (GST) and human CYP2E1 show enhanced resistance to mixed contaminates of heavy metals and organic pollutants, J. Hazard. Mater. 189 (2011) 357–362.
[19] S. Chu, C. Yang, X. Xu, X. Liu, Polychlorinated biphenyl congener residues in sediment and soil from pollution area, China Environ. Sci. 15 (1995) 199–203.
[20] J.Y. Zhang, L.M. Qiu, J. He, Y. Liao, Y.M. Luo, Occurrence and congeners specific of polychlorinated biphenyls in agricultural soils from Southern Jiangsu, J. Environ. Sci. China 19 (2007) 338–342.
[21] R. He-jun, L. Yang, Z. Rui, D. Chun-yan, W. Yan, Z. Lan-ying, Molecular cloning and characterization of a new cold-active extradiol dioxygenase from a metagenomic library derived from polychlorinated biphenyl-contaminated soil, Chem. Res. Chin. Univ. 28 (2012) 666–671.
[22] Y. Lu, Y. Yu, R. Zhou, W. Sun, C. Dai, P. Wan, L. Zhang, D. Hao, H. Ren, Cloning and characterisation of a novel 2,4-dichlorophenol hydroxylase from a metagenomic library derived from polychlorinated biphenyl-contaminated soil, Biotechnol. Lett. 33 (2011) 1159–1167.
[23] J. Hirose, N. Kimura, A. Suyama, A. Kobayashi, S. Hayashida, K. Furukawa, Functional and structural relationship of various extradiol aromatic ring-cleavage dioxygenases of *Pseudomonas* origin, FEMS Microbiol. Lett. 118 (1994) 273–277.
[24] A. Montague, A. Ziauddin, R. Lee, W.M. Ainley, J. Strommer, High-efficiency phosphinothricin-based selection for alfalfa transformation, Plant Cell Tissue Organ 91 (2007) 29–36.
[25] A. Gulati-Sakhuja, J.L. Sears, A. Nunez, H.-Y. Liu, Production of polyclonal antibodies against Pelargonium zonate spot virus coat protein expressed in *Escherichia coli* and application for immunodiagnosis, J. Virol. Methods 160 (2009) 29–37.
[26] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[27] E. Uchida, T. Ouchi, Y. Suzuki, T. Yoshida, H. Habe, I. Yamaguchi, T. Omori, H. Nojiri, Secretion of bacterial xenobiotic-degrading enzymes from transgenic plants by an apoplastic expressional system: an applicability for phytoremediation, Environ. Sci. Technol. 39 (2005) 7671–7677.
[28] H. Xia, X. Chi, Z. Yan, W. Cheng, Enhancing plant uptake of polychlorinated biphenyls and cadmium using tea saponin, Bioresour. Technol. 100 (2009) 4649–4653.
[29] B.V. Aken, P.A. Correa, J.L. Schnoor, Phytoremediation of polychlorinated biphenyls: new trends and promises, Environ. Sci. Technol. 44 (2009) 2767–2776.
[30] H. Kawahigashi, S. Hirose, H. Ohkawa, Y. Ohkawa, Transgenic rice plants expressing human CYP1A1 remediate the triazine herbicides atrazine and simazine, J. Agric. Food Chem. 53 (2005) 8557–8564.
[31] R. Andreozzi, I. Di Somma, R. Marotta, G. Pinto, A. Pollio, D. Spasiano, Oxidation of 2,4-dichlorophenol and 3,4-dichlorophenol by means of Fe(III)-homogeneous photocatalysis and algal toxicity assessment of the treated solutions, Water Res. 45 (2011) 2038–2048.
[32] M.A. Talano, D.C. Busso, C.E. Paisio, P.S. González, S.A. Purro, M.I. Medina, E. Agostini, Phytoremediation of 2,4-dichlorophenol using wild type and transgenic tobacco plants, Environ. Sci. Pollut. Res. 19 (2012) 2202–2211.